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MECHANISMS OF RESISTANCE

Hypermutator *Pseudomonas aeruginosa* Exploits Multiple Genetic Pathways To Develop Multidrug Resistance during Long-Term Infections in the Airways of Cystic Fibrosis Patients

C. A. Colque,[a,b] A. G. Albarracín Orio,[a,b,c] S. Feliziani,[a,b] R. L. Marvig,[d] A. R. Tobares,[a,b] H. K. Johansen,[e,f] S. Molin,[g] A. M. Smania[a,b]

[a]Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Departamento de Química Biológica Ranwel Caputto, Córdoba, Argentina
[b]CONICET, Universidad Nacional de Córdoba, Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Córdoba, Argentina
[c]IRNASUS, CONICET, Universidad Católica de Córdoba, Facultad de Ciencias Agropecuarias, Córdoba, Argentina
[d]Center for Genomic Medicine, Rigshospitalet, Copenhagen, Denmark
[e]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
[f]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[g]Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Lyngby, Denmark

ABSTRACT *Pseudomonas aeruginosa* exploits intrinsic and acquired resistance mechanisms to resist almost every antibiotic used in chemotherapy. Antimicrobial resistance in *P. aeruginosa* isolates recovered from cystic fibrosis (CF) patients is further enhanced by the occurrence of hypermutator strains, a hallmark of chronic infections in CF patients. However, the within-patient genetic diversity of *P. aeruginosa* populations related to antibiotic resistance remains unexplored. Here, we show the evolution of the mutational resistome profile of a *P. aeruginosa* hypermutator lineage by performing longitudinal and transversal analyses of isolates collected from a CF patient throughout 20 years of chronic infection. Our results show the accumulation of thousands of mutations, with an overall evolutionary history characterized by purifying selection. However, mutations in antibiotic resistance genes appear to have been positively selected, driven by antibiotic treatment. Antibiotic resistance increased as infection progressed toward the establishment of a population constituted by genotypically diversified coexisting sublineages, all of which converged to multidrug resistance. These sublineages emerged by parallel evolution through distinct evolutionary pathways, which affected genes of the same functional categories. Interestingly, *ampC* and *ftsI*, encoding the β-lactamase and penicillin-binding protein 3, respectively, were found to be among the most frequently mutated genes. In fact, both genes were targeted by multiple independent mutational events, which led to a wide diversity of coexisting alleles underlying β-lactam resistance. Our findings indicate that hypermutators, apart from boosting antibiotic resistance evolution by simultaneously targeting several genes, favor the emergence of adaptive innovative alleles by clustering beneficial/compensatory mutations in the same gene, hence expanding *P. aeruginosa* strategies for persistence.

KEYWORDS *Pseudomonas aeruginosa*, hypermutability, multidrug resistance, *ampC*, *ftsI*, cystic fibrosis

Antibiotic resistance has emerged as a global health concern with serious economic, social, and political implications. Accordingly, it is becoming widely accepted that we are close to a postantibiotic era due to the increasing occurrence of multidrug-resistant (MDR) pathogens and the failure to compensate for this phenomenon with drug discovery (1).

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Address correspondence to A. M. Smania, asmania@fcq.unc.edu.ar.

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Among high-risk pathogens, *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections and the third most common bacterium isolated from infections acquired in intensive care units (2). Likewise, *P. aeruginosa* chronically infects the airways of cystic fibrosis (CF) patients and constitutes their main cause of morbidity and mortality (3).

The effective intrinsic and acquired mechanisms of *P. aeruginosa* resistance to different types of antibiotics (4) and the emergence of MDR clones (5) severely compromise the treatment of these infections. Notably, several resistance genes, including genes for different classes of carbapenemases, have spread among an increasing number of *P. aeruginosa* clones through horizontal gene transfer. In many cases, this makes colistin and, to some extent, amikacin the only drugs available to treat MDR *P. aeruginosa* infections (2).

Intrinsic mechanisms of resistance involve mutations in chromosomal genes leading to the inactivation of the carbapenem porin OprD, the overexpression of AmpC, and the upregulation of efflux pumps (4, 6, 7). Importantly, the concomitant accumulation of these mutations can lead to the emergence of MDR strains, which constitute a major concern in the clinical setting (5).

Frequently, the acquisition of these adaptive mutations is enhanced by increments in mutation rates, like those observed in hypermutator strains of *P. aeruginosa*. Thus, it has been reported that 22 to 54% of chronically infected CF patients are infected with hypermutator strains of *P. aeruginosa*, which are deficient in the DNA mismatch repair (MMR) system (8–14). The hypermutator phenotype has been correlated with the increased development of antibiotic resistance (8, 12, 15–17), the acquisition of chronic infection-adaptive variants (17–20), as well as metabolic adaptive transformations (21).

Recent advances in whole-genome sequencing (WGS) techniques have provided insights into the evolutionary trajectories of adaptation of *P. aeruginosa* to the CF patient environment, particularly with regard to pathoadaptive mutations, such as those associated with antibiotic resistance (11, 22–27). In this sense, "the mutational resistome" was recently defined as the set of mutations involved in the modulation of antibiotic resistance levels in the absence of horizontal gene transfer (28, 29).

In a previous investigation, we studied the evolutionary trajectories of *P. aeruginosa* hypermutator lineages in long-term chronic infection in CF patients (25). Comparative WGS analyses showed extensive within-patient genomic diversification, with populations being composed of different sublineages that had coexisted for many years since the initial colonization of the patient. Importantly, certain genes were particularly enriched for mutations and underwent convergent evolution across the sublineages, suggesting that they are involved in the optimization process of *P. aeruginosa* pathogenic fitness. Here, we characterized the mutational resistome and the antibiotic susceptibility profile of a hypermutator lineage sampled throughout a period of 20 years of evolution from the airways of a CF patient. To gain a comprehensive picture of the evolution of antibiotic resistance, we performed a longitudinal analysis by exploring WGS data for three sequentially isolated clones and a transversal study on a collection of 11 isolates obtained from a single sputum sample, which provided a snapshot of the genetic diversity at the population level.

RESULTS

Emergence of multidrug-resistant isolates in the CFD collection. In our previous study, we sequenced the whole genomes of 14 isolates belonging to the same clonal lineage of *P. aeruginosa*, spanning 20 years of the infection history of a patient (referred to as patient CFD) (25). This collection included a normomutator isolate obtained in 1991, which we used as the ancestral reference; 2 hypermutator isolates, 1 from 1995 and the other from 2002; and 11 isolates obtained from the same sputum sample in 2011.

All the hypermutator isolates harbored the same *mutS* mutation, which inactivates the MMR system (25). As shown in Fig. 1, patient CFD received prolonged treatment with a large and varied set of antibiotics during the course of chronic infection between 1986 and 2012. Treatment included five classes of antibiotics: β-lactams, aminoglyco-

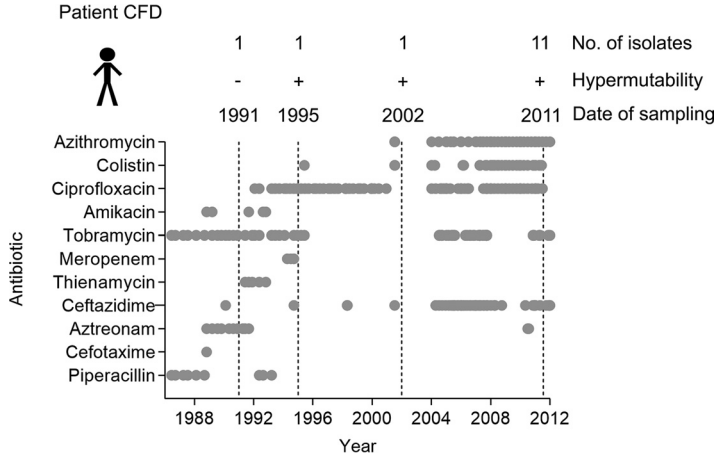

FIG 1 Overview of isolate sampling time points and antibiotic treatment. *P. aeruginosa* isolates were collected from patient CFD between 1991 and 2011. The plus and minus symbols indicate the hypermutability state of the *P. aeruginosa* strains. The antibiotics used in chemotherapy over the 20 years study are listed on the *y* axis. Gray circles indicate the start and end of an antibiotic dose.

sides, quinolones, polymyxins, and macrolides. To investigate the impact of antibiotic treatment on the resistance profiles of the CFD isolates, susceptibility to antibiotics representing all these major classes was tested by the agar diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. As observed in Fig. 2, all isolates except the 1991 isolate showed multidrug resistance, meaning a reduced susceptibility to two or more classes of antibiotics. Starting from the general susceptible phenotype of the 1991 isolate, the 1995 isolate exhibited resistance to β-lactams, whereas the 2002 isolate, in addition to resistance to β-lactams, gained resistance to ciprofloxacin, tobramycin, and colistin. Importantly, the collection of 2011 isolates showed the highest levels of resistance to ciprofloxacin, tobramycin, azithromycin, colistin, and, particularly, β-lactams, such as cephalosporins and the monobactam aztreonam. Interestingly, in contrast to the 1995 isolate, the 2002 isolate and all the 2011 isolates showed susceptibility to piperacillin-tazobactam, resistance to which seems to have been lost after the acquisition of resistance to tobramycin, thus suggesting collateral sensitivity to penicillin-type β-lactams, as previously described by Barbosa et al. (30). On the other hand, even though colistin was used from 2004, all

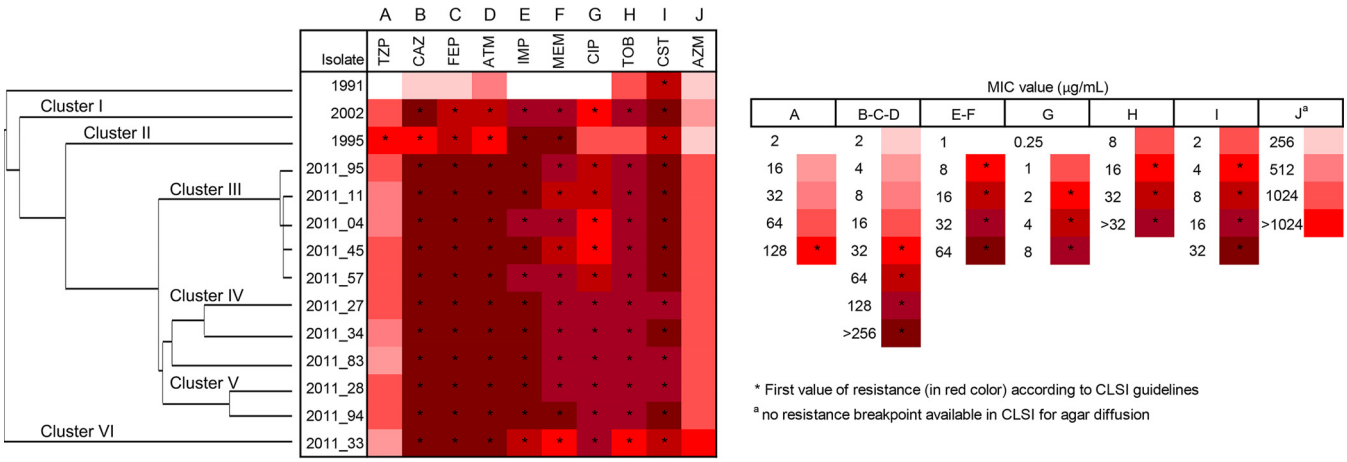

FIG 2 Antibiotic resistance profiles of *P. aeruginosa* isolates from the CFD lineage. Each column represents the MIC values of the different antibiotics tested: piperacillin-tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IMP), meropenem (MEM), ciprofloxacin (CIP), tobramycin (TOB), colistin (CST), and azithromycin (AZM). The red intensity indicates the MIC levels for each antibiotic. Asterisks indicate resistance according to CLSI guidelines. The phylogenetic tree on the left represents the genetic clustering of isolates (rows), based on the result of maximum-parsimony analysis, and was constructed based on the accumulation of new SNPs relative to the sequences of the ancestor from 1991 (25).

isolates showed MIC values ranging from 8 to 32 μg/ml, which are relatively high compared to those seen in other data sets from clinical isolates (28, 29, 31, 32) (Fig. 2). Finally, although *P. aeruginosa* has no clinical breakpoints established for azithromycin, the evolved isolates showed relatively higher MICs than the 1991 and 1995 isolates.

Mutations for antibiotic resistance are positively selected during evolution. In order to investigate the molecular bases of the antimicrobial resistance observed in the CFD isolates, we explored the acquisition of mutations in a set of 168 chromosomal genes, here defined as the resistome, which have been described to be involved in *P. aeruginosa* antibiotic resistance mechanisms (28, 29, 33). Thus, using the 1991 genome sequence as a reference, we analyzed the distribution of a total of 5,710 single-nucleotide polymorphisms (SNPs) and 1,078 indels, which we have previously detected in the collection of isolates by WGS analysis (25), that accumulated over a period of 20 years of infection. Furthermore, we also analyzed 39 SNPs (26 synonymous SNPs, 13 nonsynonymous SNPs) detected in the genome sequence of the 1991 isolate compared to the PAO1 genome sequence. Sequence variations found within the resistome are documented in Table S1 at https://www.researchgate.net/publication/339487394_Supplementary_Material_AAC02142-19pdf. Interestingly, 96 (57%) of the 168 investigated genes showed nonsynonymous SNPs and/or indel mutations of 1 to 3 bp in at least one of the isolates. On the other hand, 11 (6%) genes showed only synonymous mutations, and 61 (36%) showed no mutations (see Fig. S1 at https://www.researchgate.net/publication/339487394_Supplementary_Material_AAC02142-19pdf). Furthermore, 90 out of the 91 genes harboring nonsynonymous mutations (99%) were targeted with missense mutations, whereas a single gene (1%) showed a nonsense mutation (Fig. S1 and Table S1). By analyzing the ratio between nonsynonymous and synonymous mutations (*dN*/*dS* ratio) within the resistome in each CFD isolate, we observed that in most isolates the signature of selection was higher than 1 (see Table S2 at https://www.researchgate.net/publication/339487394_Supplementary_Material_AAC02142-19pdf). This indicates that these mutations were positively selected during chronic infection and suggests that hypermutability may be linked to them as a key factor contributing to antibiotic resistance in CF patients. Mutational resistome analysis was further focused on those genes that were targeted with nonsynonymous and/or frameshift mutations (Fig. 3), showing that the accumulation of these mutations correlated with increased antibiotic resistance. Moreover, no mutations were detected in the ancestral 1991 isolate with respect to the sequence of reference strain PAO1, in agreement with its general antibiotic susceptibility (Fig. 2). In some genes known to be involved in antibiotic resistance, single mutations were identified, such as D87G in GyrA and S278P in OprD (34, 35); in others, the accumulation of 3 to 5 different mutational events suggests that they have evolved under strong selective pressure. Such examples are *amgS*, *mexX*, and *fusA2* (29, 36) (involved in aminoglycoside resistance), *mexF*, *oprN*, *poxB*, and *mexI* (involved in β-lactam resistance), *polB*, *mexD*, and *parE* (involved in quinolone resistance), *spuF* (part of the polyamine uptake system, which is involved in β-lactam resistance by avoiding polyamine adjuvant activity against these antibiotics) (37–39), and *mexK* (coding for a novel efflux system, MexJK) (40). Remarkably, *mexY* and *fusA1* (involved in aminoglycoside resistance) and *ampC* and *ftsI* (involved in β-lactam resistance) accumulated more than 6 different mutations (Fig. 3). Given a Poisson distribution probability of $P(X \geq 6) \sim \text{pois}(\lambda = 1.02) = 0.00066$, where λ is the expected number of mutations per gene and X is the observed number of mutations in the gene, it would be expected that 0.11 of the 168 genes of the resistome would acquire $\geq$6 mutations. Then, having 4 genes with $\geq$6 mutations represents an occurrence 36-fold higher than what would be expected by chance and represents a significant increase in the mutation density in these genes, providing strong evidence for positive selection.

The β-lactam resistome. As shown in Fig. 1, patient CFD received prolonged antibiotic treatment courses with ceftazidime as well as shorter courses of various durations with different types of β-lactams, including other cephalosporins (cefotaxime), penicillins (piperacillin and tazobactam), monobactams (aztreonam), and car-

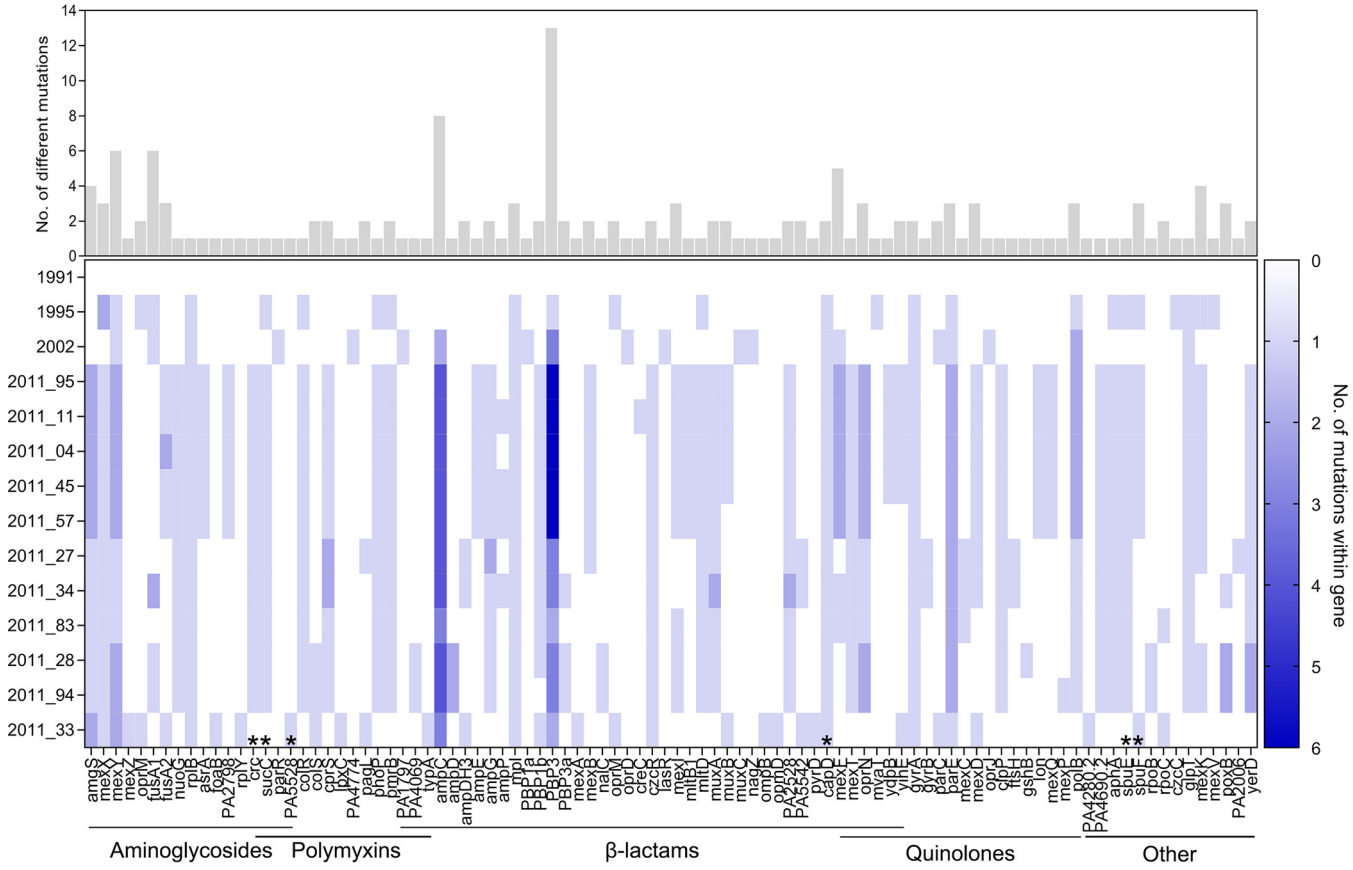

FIG 3 Resistome of the CFD isolate collection. Mutations potentially affecting protein function for 93 out of the 168 antibiotic resistance genes were analyzed. Genes and variants were grouped by antibiotic class. (Top) Number of independent mutations found within each specific gene along the CFD lineage. (Bottom) Heat map of the number of mutations accumulated per gene in each genome. Isolates were grouped based on the genetic clustering defined in Fig. 2. Asterisks indicate genes which are also involved in conferring resistance to other antibiotic classes: *crc* (β-lactams), *sucC* and *PA5528* (quinolones), *capD* (aminoglycosides), and *spuE* and *spuF* (β-lactams).

bapenems (thienamycin and meropenem). As expected, resistance increased from the 1995 isolate to later isolates, with the highest levels of resistance to cephalosporins, aztreonam, and carbapenems being reached in the 2011 isolates (Fig. 2). A total of 70 genes have been reported to be involved in the β-lactam resistome (28, 29), including genes encoding the regulation of peptidoglycan recycling (responsible for AmpC overproduction); genes encoding penicillin-binding proteins (PBPs; targets of β-lactam antibiotics); genes encoding regulators of efflux pumps, such as MexAB-OprM (involved in β-lactam resistance) and MexEF-OprN (involved in carbapenem resistance); and the *oprD* gene (involved in resistance to imipenem and susceptibility to meropenem). We found that 42 of these 70 genes (60%) showed nonsynonymous and/or frameshift mutations in at least one isolate of the CFD collection (Fig. 3). Of these 42 genes, 36 showed accumulations of 1 to 2 mutations, with most of them being unique to each specific cluster. It has been described that the emergence of resistance to penicillins and cephalosporins is mainly due to overproduction of the β-lactamase AmpC (41). However, the most frequent drivers of AmpC overproduction described in *P. aeruginosa* clinical strains, namely, *ampD*, *ampR*, and *dacB* (42–44), were not mutated among the CFD isolates. Instead, all but the 1991 isolate showed a frameshift mutation in *mpl*, which encodes a UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase, indicating that *ampC* could be overexpressed via this alternative negative regulator (45, 46). Western blot analyses showed that all 2011 isolates showed an increased expression of AmpC compared to the 1991, 1995, and 2002 isolates, suggesting that alternative pathways may be responsible for AmpC overproduction in

these isolates (see Fig. S2 at https://www.researchgate.net/publication/339487394_Supplementary_Material_AAC02142-19pdf). In addition, *ampC* was among the most mutated genes in the CFD collection, together with the *ftsI* gene, with these two genes showing 8 and 13 distinct missense mutations, respectively (Fig. 3). In fact, all CFD isolates, except those from 1991 and 1995, showed the accumulation of mutations within *ampC*, with isolates from 2011 carrying up to four different mutations combined into newly reported, not previously reported, *ampC* alleles. This strongly correlates with the increase in the MICs of cephalosporins and with aztreonam resistance in the evolved CFD isolates (Fig. 2), indicating that they were under high selective pressure during the CFD chronic infection process. Interestingly, the presence of mutations such as P154L, G216S, and V213A has been reported to be involved in β-lactam resistance (47), and H189Y, V213A, and V330I have been shown to emerge *in vivo* during chronic infections in CF patients (11, 14, 29). A study enabling a deep understanding of the contribution of these new *ampC* alleles to the β-lactam resistance profile is currently in progress in A. M. Smania's laboratory. Likewise, all isolates except for the isolate from 1991 showed mutations in *ftsI*, with the isolates from 2011 showing up to six mutations combined in a single allele. Some of these mutations (Y367C, H394R, N427S, Q458R, Q475R, R504L, V523A, V523M, and F533L) are located in the transpeptidase β-lactam binding site of the protein, and the F533L mutation has been shown to play a key role in β-lactam recognition (48). Importantly, these mutations have been documented to emerge among CF patient *P. aeruginosa* isolate collections (29, 49, 50) and upon aztreonam (51), ceftazidime (52), and meropenem (53) exposure *in vitro*, whereas other mutations are described for the first time in this work (Table S1). The rest of the PBP-encoding genes showed few mutations among the CFD collection.

Although patient CFD received only short courses of treatment with carbapenems, we observed the emergence of high levels of resistance to carbapenems in all isolates except for the ancestral 1991 isolate. Previous reports have shown that loss-of-function mutations in the outer membrane protein OprD and/or overexpression of the efflux pumps MexAB-OprM (resulting in meropenem resistance) and MexEF-OprN (resulting in imipenem and meropenem resistance) constitutes the main mechanism for the development of carbapenem resistance. However, only the 2002 isolate (cluster I, defined according the phylogenetic tree shown in Fig. 2) showed a missense mutation (S278P) within the *oprD* gene, which has previously been described to be involved in carbapenem resistance (35). Expression of the MexAB-OprM system is controlled by the regulatory genes *mexR*, *nalC*, and *nalD* (54, 55), and a missense mutation in *nalC* (M151T) was identified in the two isolates from cluster V. Additionally, mutations in the two-component system CzcRS, which contribute to zinc, cobalt, and cadmium resistance by promoting the expression of the metal efflux pump CzcCBA, downregulate the expression of the OprD porin, rendering *P. aeruginosa* resistant to both trace metals and carbapenems (56, 57). Importantly, high levels of zinc occur in CF patient sputum and promote the virulence of *P. aeruginosa* by a CzcRS-dependent mechanism that controls *oprD* expression and that modifies the carbapenem susceptibility profile (58). Indeed, whereas isolate 2011_33 carried the D180N mutation in CzcR, the rest of the 2011 isolates harbored a V48A mutation, located in the response regulator domain of the transcriptional regulator. Nevertheless, whether these mutations contribute to *czcR* overexpression and contribute to either meropenem or imipenem resistance needs to be further characterized. As mentioned above, the mutations F533L and R504 in PBP3 have been found to occur upon meropenem exposure during *in vitro* evolution studies and among CF patients treated with this drug (50, 53). Thus, high levels of carbapenem resistance may also be associated with the presence of these *ftsI* mutations. Importantly, using the ResFinder tool on WGS data from the CFD isolates, we did not find genes coding for any class of metallo-β-lactamases (MBLs) involved in carbapenem resistance, which are normally acquired through horizontal gene transfer (59). These results suggest that various different mutational mechanisms may be involved in carbapenem resistance in different coexisting CFD isolates, giving rise to distinct genetic pathways for the evolution of resistance to β-lactams.

The aminoglycoside resistome. As shown in Fig. 1, patient CFD received extensive courses of tobramycin treatment. MIC determinations showed that all isolates, except for the 1991 and 1995 isolates, became resistant to tobramycin. The main origin of high-level resistance to aminoglycosides is overexpression of the MexXY-OprM efflux system (60), which is primarily caused by *mexZ* mutations (10, 22, 61). In addition, mutations in the *amgRS* and *parRS* two-component system genes have also been involved in the regulation of MexXY expression (62). No *mexZ* mutations, however, were observed in the CFD collection of isolates, with the sole exception of isolate 2011_33 (cluster VI), which showed a V29A mutation, which is located within the DNA binding domain of the protein (Table S1) (63) and which is predicted to be deleterious (score of −3.351, determined with the PROVEAN tool, v1.1.3). Instead, we found four different mutations in the *amgS* gene, encoding the histidine kinase sensor of the membrane stress response two-component system; six mutations in *mexY*, encoding a component of the MexXY efflux pump; and six mutations in *fusA1*, which codes for elongation factor G.

MexY mutations have been frequently observed among drug-resistant isolates and CF patient epidemic clones (29, 32). Some mutations affect general pump operation and impair the MexY-dependent aminoglycoside resistance, whereas other mutations located in domains associated with aminoglycoside recognition and export may improve drug accommodation and, consequently, increase resistance (64). Furthermore, it was observed that the MexY mutation F1018L is able to increase pump-promoted resistance to aminoglycosides, cefepime, and fluoroquinolones (65). Importantly, here we describe for the first time the six *mexY* missense mutations. In this sense, their impact on MexXY pump function and aminoglycoside resistance remains unclear and deserves further investigation.

Mutations in the *amgS* gene have been shown to be involved in intrinsic aminoglycoside resistance in *P. aeruginosa* (66). Although none of the four mutations in *amgS* found here have been previously reported (Table S1), all isolates from 2011 except 2011_33 showed an A203V mutation located within the linker HAMP domain (present in histidine kinases, adenyl cyclases, methyl-accepting proteins, and phosphatases). Interestingly, it has been reported that mutations in the linker domain of EnvZ, the closest *Escherichia coli* homolog of AmgS, often cause activation of the kinase sensor (67, 68). Moreover, we found the P116L mutation, predicted to be deleterious (−2.539, determined with the PROVEAN tool, v1.1.3). This mutation is located in the sensor domain of AmgS, where mutations involved in aminoglycoside resistance have been previously described (66).

FusA1 mutations have recently been linked to the emergence of aminoglycoside resistance *in vitro* (69–71) as well as in clinical CF patient strains (29, 36, 72, 73). In fact, aminoglycoside resistance seems to be an indirect consequence of the alteration of elongation factor G (70). Isolate 2011_34 harbored two substitutions, V93A and D588G, located in domains G and IV of the protein, respectively, which have been reported to be gain-of-function mutations (29). Indeed, the V93A mutation was found to increase resistance to several aminoglycosides, such as tobramycin, amikacin, and gentamicin (70). In several CFD isolates, we identified four novel mutations in the *fusA1* gene across the different domains of the protein sequence: domain II (V338A), domain III (A481V), domain IV (A595V), and domain V (Y683C). Isolate 1995, harboring the Y683C mutation, showed susceptibility to tobramycin (Fig. 2), suggesting that this mutation is not involved in aminoglycoside resistance. Moreover, Bolard et al. (70) recently reported that higher MICs are associated with mutations in domains II, IV, and V but not with mutations in domains G and III. Therefore, only the mutations V338A (in the isolate from 2002) and A595V (in 2011 isolates from cluster III; Fig. 2) are expected to contribute to aminoglycoside resistance, although the effect of both substitutions needs to be characterized in future works. In conclusion, the high level of aminoglycoside resistance in the CFD population seems to have been acquired mostly by different mutations in the *amgS* and/or *fusA1* gene.

The fluoroquinolone resistome. Patient CFD received two prolonged periods of treatment with ciprofloxacin, from 1992 to 2002 and from 2004 to 2012 (Fig. 1). The MICs of ciprofloxacin revealed that most of the isolates exhibited high levels of resistance to this antibiotic, whereas the 1991 and 1995 isolates showed susceptibility and intermediate resistance, respectively (Fig. 2). A high level of resistance to ciprofloxacin usually involves one or several mutations in the quinolone resistance-determining (QRD) regions of the GyrAB subunits of topoisomerase II (gyrase) and the ParCE subunits of topoisomerase IV (29). Indeed, all CFD isolates except for the 1991 isolate harbored the same D87G mutation in GyrA. In addition, isolate 2011_33 also carried a T83I mutation in this gyrase subunit. Importantly, both mutations are known to be involved in quinolone resistance (29, 32, 34, 74). Furthermore, two 2011 isolates from cluster IV harbored an S618L substitution in GyrB. On the other hand, all isolates except for the 1991 isolate accumulated mutations in the topoisomerase IV subunits ParC (P308L, T705A) and ParE (V199M, D462G, S492F), none of which have been previously described. Whether these mutations that clustered in the chromosomally encoded topoisomerases II and IV were involved in quinolone resistance or were randomly fixed by genetic drift upon the high mutation supplies provided by hypermutability remains to be elucidated. Nevertheless, the fact that many different mutations arose after fluoroquinolone treatments supports the previous observation that mutations involved in fluoroquinolone resistance can be highly variable (29). Importantly, with the use of the ResFinder tool, we found the acquisition of a novel plasmid-encoded ciprofloxacin-modifying gene encoding the enzyme CrpP (75), which may explain the high-level resistance profile observed in the two intermediate isolates from 1995 and 2002 and all isolates from 2011.

Finally, we noticed that no mutations in the negative regulator *nfxB*, which is commonly reported to achieve resistance to ciprofloxacin in a CF context due to the deregulation and concomitant overexpression of the efflux pump MexCD-OprJ (76), were observed among the CFD isolates. Furthermore, although all cluster IV isolates from 2011 harbored a nonsense mutation in the transporter MexD (W1023STOP), which inactivates the efflux pump, it has been described that this mutation has no effect on the MIC of ciprofloxacin (77).

The polymyxin resistome. Patient CFD received intensive treatment with colistin from 2004 to 2011 (Fig. 1). According to CLSI, antibiotic susceptibility profiling revealed that every CFD isolate was resistant to colistin (Fig. 2). However, the evolved 2011 isolates from clusters III, IV, and V, as well as the 2002 isolate, showed 2- to 4-fold increases in their MICs relative to those of the 1991 and 1995 isolates (8 μg/ml) (Fig. 2). Clinical strains of *P. aeruginosa* sometimes show resistance to polymyxins due to mutations in different two-component systems, such as PhoPQ, PmrAB, ParRS, CprRS, and ColRS (78–82). Additionally, mutations causing derepression of the lipopolysaccharide (LPS)-modifying (*arn*) operon, encoding the proteins necessary for the aminoarabinosylation of the lipid A moiety of the LPS, have been identified in colistin-resistant *P. aeruginosa* strains (31, 80, 83). As shown in Fig. 3, the different CFD isolates accumulated unique mutations in the *phoP*, *pmrB*, *parR*, *colR*, and *colS* genes, which may affect each of the mentioned two-component systems. In fact, the 2002 isolate harbored an A45T mutation in ParR, located in the receiver domain and close to the conserved phosphorylation residue D57, which was previously shown to be involved in colistin resistance (84). On the other hand, considering that mutations V30A in PhoP and D138N in ColR were present in the more susceptible 1995 isolate, the increased resistance observed in the 2011 evolved isolates from clusters III, IV, and V could be explained by the presence of mutations in PmrB (T132A), CprS (G396S), and/or ColS (T138A). These novel mutations are the first to suggest their contribution to polymyxin resistance and therefore need to be further explored.

Other antibiotics. From the beginning of 2004 to 2012, patient CFD received systematic long-term treatments with azithromycin combined with other antipseudomonal agents. Although macrolide resistance is frequent among CF isolates, only two

reports have described the emergence of macrolide resistance *in vivo* (33, 85). As shown in Fig. 2, the MICs of azithromycin for the later isolates within the 2011 collection showed a 4-fold increase or more relative to those for the ancestral isolate from 1991. Consistent with this, all 2011 isolates carried mutations in the gene *PA4280.2*, which encodes the 23S ribosomal subunit. In fact, isolate 2011_33 carried an A2044G substitution, whereas the remaining isolates from 2011 carried a C2597T mutation; both of these are located in the secondary structure of domain V of the rRNA gene and have previously been reported to confer macrolide resistance (33, 85). Thus, macrolide resistance in the coexisting 2011 CFD isolates was acquired by distinct mutations in the same gene. This provides additional evidence for parallel molecular evolution at the population level, with antibiotic chemotherapy being the key selection force during long-term chronic infections in CF patients.

DISCUSSION

The high prevalence of hypermutator clones in CF patient chronic infections is a matter of great relevance because their link to antibiotic resistance hampers infection management (8–10, 15, 25, 86). In this study, we explored the evolution of the mutational antibiotic resistome of a *P. aeruginosa* hypermutator lineage by combining a longitudinal analysis and a transversal analysis that covered 20 years of chronic infection in a CF patient. Antibiotic resistance increased as infection progressed toward the establishment of a population consisting of genotypically diversified coexisting sublineages, all of which converged to multidrug resistance. Particularly, while the mutations observed in *amgS* most likely altered MexXY pump regulation (87), mutations affecting other multidrug efflux pump regulators were only rarely observed among the CFD isolates. Instead, multidrug resistance emerged through the combination of multiple resistance mutations in several independent loci.

Hypermutators can be indirectly selected for and fixed by their genomic association with fitness-improving alleles (88–91). Particularly, under selective conditions imposed by long-term antibiotic therapy in the CF airways, *de novo* beneficial mutations can be expected to accumulate over time. Early mutational events occurring during the course of long-term infection are expected to have a strong impact on the resistance phenotype and, consequently, on fitness. Later mutations, many of which are compensatory, may lead to the fine-tuning of the activity/stability of the resistance-related proteins, in which epistatic interactions may play important roles for the trajectories of resistance development (92–95). *P. aeruginosa* carries many different genes which, upon functional mutation, provide a resistance phenotype (28, 29). Identification of these genes and the associated polymorphisms involved in resistance document how many of them converge through distinct genetic pathways to the same or similar resistance profiles (27, 96, 97).

Hypermutability increases the likelihood of reaching the most appropriate combinations in adaptive terms. Considering the multiple genetic pathways in *P. aeruginosa* behind different resistance mechanisms, our observations show how hypermutability increases the probability of exploiting these distinct pathways, which eventually converge toward multiantibiotic resistance in the course of long-term chronic infections.

We show how aggressive and persistent chemotherapy targeting a hypermutator population resulted in repeated but independent mutagenic events in resistance-associated genes, providing clear evidence of parallel evolution in clones of the CFD population. This was, for example, the case with *ampC* and *ftsI*, which for the CFD lineage constituted hot spots for the accumulation of mutations involved in β-lactam antibiotic resistance (44, 47, 53, 59). Some of these mutations have been previously described, whereas others are reported here for the first time. Most interestingly, novel alleles were observed, with each one harboring a combination of 2 to 6 mutations.

Overproduction of the β-lactamase AmpC is considered to be the main cause of resistance to first- and second-generation cephalosporins as well as aminopenicillins in *P. aeruginosa* clinical strains (41). However, *P. aeruginosa* is also able to adapt to new

and more effective β-lactams (98) through a variety of mutations affecting the AmpC β-lactamase (47, 53, 99). Here, we document the confluence of both strategies: variants overproducing AmpC, in which the combination of distinct mutations may contribute to even higher levels of resistance and/or substrate spectrum extension. Furthermore, the accumulation of several different mutations in the penicillin-binding protein PBP3 may be a complementary and/or additional pathway. PBP3 relevance in β-lactam resistance, including the new-generation cephalosporins and carbapenems, has been very recently confirmed (28, 29, 32, 49, 53). The high number of different mutations clustered in both the *ampC* and *ftsI* genes combines into innovative resistance-conferring alleles, which demonstrates how drug resistance mutations can become highly beneficial when combined with compensatory mutations, and thus documents the extraordinary ability of *P. aeruginosa* to develop antibiotic resistance, which limits our available therapeutic arsenal.

How can we understand the coexistence of many different genetic variants showing the same resistance profile in patient airways, as has been documented here? One answer is based on the balance between clonal interference and multiple mutations (100, 101). We thus argue that hypermutability increases the rate of antibiotic resistance evolution by increasing the piggybacking of multiple resistance mutations, causing maintenance of a diversified population where adaptive variation is sustained by a dynamic equilibrium between mutation and selection. Moreover, in long-term evolutionary scenarios, such as chronic infections, the selective forces imposed by antibiotics, along with the high mutation rates from hypermutability, may shape genetically diverse populations able to respond successfully to antibiotic treatments, ensuring the persistence of the bacterium. These observations may put clinicians from diagnostic laboratories on the alert about antimicrobial susceptibility testing, which is frequently carried out on single isolates, thus affecting its efficacy as a predictor. Furthermore, the fact that apparently equivalent multidrug-resistant phenotypes can be based on many different mechanisms may lead to erroneous treatments and antibiotic therapy failures.

Our results provide new evidence concerning the way in which hypermutators can expedite the evolution of multidrug resistance by increasing the probability of acquiring adaptive mutations to support the long-term survival of *P. aeruginosa* in the airways of CF patients.

MATERIALS AND METHODS

***P. aeruginosa* CFD collection.** Clinical *P. aeruginosa* isolates were obtained from sputum samples from a CF patient attending the Copenhagen CF Centre at Rigshospitalet (Copenhagen, Denmark) (patient CFD). In a previous study (25), we sequenced the genomes of 14 isolates from this patient covering ~20 years of the patient's life span. The CFD collection included 1 normomutator isolate obtained in 1991 (CFD_1991), 5 years after the onset of chronic *P. aeruginosa* infection in 1986; 2 sequential mismatch repair (MRS)-deficient mutators from 1995 (CFD_1995) and 2002 (CFD_2002) that harbored the same ΔCG mutation in *mutS* at position 1551; and 11 *P. aeruginosa* isolates obtained from a single sputum sample in 2011 (CFD_2011), all harboring the ΔCG *mutS* mutation at position 1551 and belonging to the same hypermutator lineage.

Ethics statements. The *P. aeruginosa* isolates were obtained from sputum samples from one CFD patient at the Copenhagen CF Centre at Rigshospitalet (Copenhagen, Denmark) (patient CFD), as by-products of the routine established for bacterial typing and antimicrobial susceptibility testing. That is, sputum sampling was not performed for the purposes or intent of the present study; isolates recovered from these sputa were simply derivatives of routine CF patient therapeutic controls. The therapeutic treatments of the patient were not modified in any way as a consequence of the results obtained in this study. The research protocols followed in this study were by the local ethics committee, Region Hovedstaden, Copenhagen, Denmark (H-A-141 and H-1-2013-032). The patients gave informed consent.

Genome sequencing analysis. Detailed information on the sequencing/assembly methodologies, the parameters used for calling variants, and standard quality control metrics can be found in the work of Feliziani et al. (25). Briefly, genomic libraries were sequenced on an Illumina HiSeq2000 platform (ENA/SRA accession number ERP002379), and reads from CFD_1991 were *de novo* assembled using Velvet software (v1.2.07) (102) (selected kmer size, 39; coverage depth, 200 times; number of contigs, 601). CFD_1991 was used as a reference to map reads from the remaining CFD genome sequences using Novoalign software (v2.08.02; Novocraft Technologies) (103) and processed with the SAMtools program (v0.1.7) (104). SNPs were identified by the varFilter algorithm in SAMtools, and only unambiguous SNPs with quality scores of ≥50 were retained. Read alignments surrounding all putative indels were realigned

using the GATK (v1.0.5083) program (105). The MUMmer3 program (106) was used for whole-genome sequence alignments, and coverage depths were between 85 and 140 times. Mutations were described according to their relative gene positions in orthologs of the complete genome sequences of the *P. aeruginosa* reference strains PAO1, PA14, and LESB58.

Profiling of antibiotic resistance genes. In order to correlate the documented resistance genotypes with the observed resistance phenotypes, single-nucleotide polymorphisms (SNPs) and indels (1- to 10-bp insertion/deletion mutations) for each isolate obtained from the previous study (25) were filtered based on an exhaustive literature review (28, 29). We also added to the list the *PA0668.4*, *PA4280.2*, *PA4690.2*, and *PA5369.2* genes, affecting macrolide resistance (33). Thus, we obtained a set of 168 genes known to be related to antibiotic resistance in *P. aeruginosa*. Indels and premature stop codons were considered to result in the inactivation of the corresponding protein product. The contribution of the documented SNPs to the phenotype was evaluated according to the available literature and by using online software tools for prediction of the effect of nucleotide substitutions on protein function, e.g., SIFT (107), PROVEAN (108), and SNAP2 (109). In addition, the online tool ResFinder (v2.1; https://cge.cbs.dtu.dk//services/ResFinder/) (110) was used to identify possible horizontally acquired antimicrobial resistance genes.

Susceptibility testing. MIC determinations were performed by using the broth dilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines and breakpoints (111). Ten antimicrobial agents from five classes of antibiotics were tested (the values in parentheses are the MIC breakpoint for susceptible/MIC breakpoint for resistance): from the β-lactam class, ceftazidime (8/32 μg/ml), cefepime (8/32 μg/ml), piperacillin-tazobactam (16 to 4/128 to 4 μg/ml), aztreonam (8/32 μg/ml), imipenem (2/8 μg/ml), and meropenem (2/8 μg/ml); the aminoglycoside tobramycin (4/16 μg/ml); the fluoroquinolone ciprofloxacin (0.5/2 μg/ml); the polymyxin colistin (2/4 μg/ml); and the macrolide azithromycin (for which no breakpoint information is available). *P. aeruginosa* ATCC 27853 was used as the quality control strain.

AmpC expression levels. CFD isolates were grown for 16 h on LB medium, and 1.5 ml of each culture was pelleted; resuspended in 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 15% glycerol amended with 0.2-mg/ml lysozyme, 1 mM 8 phenylmethylsulfonyl fluoride, and 1 mM benzamidine; and incubated for 1 h on ice. After four sonication (2 min) and freeze-unfreeze cycles, intact cells were removed by centrifugation at 9,000 $\times$ *g* for 20 min and the extracts were stored at -20°C. Twenty-five micrograms of total proteins was separated through sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) 12% gels, and then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 1.5 h at 350 mA. The blots were blocked for 1 h in 5% milk in phosphate-buffered saline (PBS) solution at room temperature. Incubation with primary antibody (rabbit anti-PDC-3 polyclonal antibody) (112), which was added at a 1/1,000 dilution, was performed overnight at 4°C in 5% milk–PBS, and then washings were performed with PBS-Tween 20 and the secondary antibody (IRDye 680RD anti-rabbit; LI-COR Bioscience), which was added at a 1:20,000 dilution for 1 h in 5% milk–PBS. Membranes were scanned on Odyssey infrared imager instrument (LI-COR Bioscience).

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